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Effect of Apoptosis on Porcine Parthenotes Development in vitro

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돼지 단위발생 배아의 발달과정에서 세포사멸에 관한 연구

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Abstract This study was conducted to determine the effects of fetal bovine serum (FBS), bovine serum albumin (BSA) and epidermal growth factor (EGF) on blastocoel formation, total cell number, apoptosis and apoptosis-related gene expression of porcine diploid parthenotes developing in vitro. The addition of 0.4% BSA to the culture medium enhanced the development of 2-cell stage parthenotes to the blastocysts stage ($p<0.01$). Treatment of FBS reduced cell numbers of blastocysts ($p<0.01$) and increased the percentage of apoptosis in blastocysts ($p<0.001$). However, BSA increased cell numbers, only in the presence of EGF reverse-transcriptase polymerase chain reaction revealed that EGF enhanced the mRNA expression of Bcl-xL in the presence of 0.4% BSA but BSA and EGF alone had no effect. However, Treatment of FBS reduced Bcl-xL mRNA expression ($p<0.05$) and enhanced Bak expression. This result suggests that apoptosis related gene expression is significantly affected by supplements, which may result in alteration of apoptosis and embryo viability of porcine embryos developing in vitro.

요 약 이배체 단위발생 돼지 난자를 체외 배양시 배반포 형성 단계에서 FBS (우태아 혈청), BSA (우혈청 알부민), EGF (상피세포 성장인자)를 배양액에 첨가하였을 때 이배체 단위발생 에서 총세포수, 세포사멸 및 세포사멸에 관여하는 유전자의 발현 효과를 조사하고자 본 연구를 수행하였다. 0.4% BSA를 배양액에 첨가 하였을 때 2 세포기 단계 단위발생의 발달은 배반포 까지는 강화 되었다 ($p<0.01$). FBS 처리 시는 배반포의 세포 수는 감소시켰으나 세포 사멸률은 증가하였다 ($p<0.01$). 하지만 EGF가 존재할 때 BSA 처리는 총 세포수를 증가 시켰다. RT-PCR의 결과에 의하면 EGF는 0.4% BSA가 존재하는 배양액에서는 Bcl-xL mRNA 발현을 증가시키고 BSA와 EGF 가 단독으로 존재 할 때는 효과가 없었다. 하지만 FBS 처리시 Bcl-xL 유전자 발현은 감소하고 Bak 유전자의 발현은 증가시킨다. 이러한 결과 세포사멸에 관여하는 유전자의 발현은 배양액의 첨가물에 따라 유의적으로 영향을 받으며, 돼지 배아의 체외 배양시 세포사멸과 초기발달에 관여함을 시사한다.

Key Words : Apoptosis, Blastocyst, BSA, EGF, FBS, Porcine embryo

1. INTRODUCTION

Systems to culture mammalian embryos in vitro have been developed to elucidate early embryonic development and to produce viable embryos. The basic culture system used to develop the pre-implantation mammalian embryo employs bovine serum albumin (BSA) as a common constituent of the culture medium. BSA seems to provide beneficial factors such as energy substrates or scavenger ions and small molecules [1]. However, medium-containing BSA is still poorly defined and it contains many unknown proteins and growth factors which may affect directly or indirectly embryo development.

Preimplantation embryos express a variety of growth

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factor receptors. These include epidermal growth factor (EGF) receptors, which are expressed during the preimplantation stage by murine porcine[2]. and human embryos[3]. Due to the expression of such receptors, many aspects of embryo development can be modulated by the presence of exogenous growth factors in the culture medium. In particular, exogenous EGF enhances the developmental rate and mitogenesis of preimplantation murine and bovine embryo

Programmed cell death or apoptosis is crucially involved in development and differentiation. Environmental stresses such as those imposed during in vitro cultures—can induce unscheduled apoptosis in cultured embryos; which may lead to arrest or abnormal development and decrease in embryo viability [5]. Cory and Adams[6]. reported of the Bcl-2 gene family play key roles in regulating apoptosis. At least 15 mammalian Bcl-2 gene family members have been identified and categorized into two subgroups, anti-apoptotic (Bcl-2, Bcl-w, Bcl-xL, A1, Mcl-1) and pro-apoptotic (Bax, Bak, Box, Bik, Blk, Hrk, BNIP3, Bim, Bad, Bid, Bcl-xS). Effects of different culture systems on mRNA expression pattern of apoptosis-related genes have been determined in human[7]. mouse[8]. and bovine[6] embryos but not in porcine embryos.

It is difficult to obtain pig embryos of homogeneous quality due to the relatively high incidence of polyspermy during in vitro fertilization. Therefore, diploid parthenotes have frequently been used to study early development in the pig[9]. In the present study we determined effects of PVA, FBS, BSA and EGF on the developmental ability and apoptosis of porcine 2-cell (30h after activation) developing in vitro. We additionally determined relative amounts of gene expression of Bcl-2 and Bak in porcine parthenotes developed from same culture conditions using sensitive semiquantitative reverse transcriptase polymerase chain reaction.

2. MATERIALS AND METHODS

2.1 In vitro Porcine Oocyte Maturation and Parthenogenesis Activation

Prepubertal porcine ovaries were collected from a local slaughterhouse and transported to the laboratory at 25℃ in Dulbecco phosphate-buffered saline (PBS) supplemented with 5.54 mM D-glucose, 0.33 mM sodium pyruvate, 75 μg/ml potassium penicillin G and 50 g/ml streptomycin sulphate (mDPBS). Cumulus-oocyte complexes (COC) were aspirated from follicles 3 to 6 mm in diameter with an 18-gauge needle into a disposable 10 ml syringe. The COC were washed 3 times with HEPES buffered Tyrodes medium containing 0.1% (w/v) polyvinyl alcohol (HEPES-TL-PVA). Each group of 50 COC was matured in 0.5 ml tissue culture medium (TCM)-199 supplemented with 0.57 mM cysteine (Sigma, St Louis, MO), 10 ng/ml epidermal growth factor (EGF, Sigma), 10 IU/ml PMSG (Sigma) and 10 IU/ml hCG (Sigma) under paraffin oil at 39℃ for 44 h. Following maturation, cumulus cells were removed by pipetting in the presence of 1 mg/ml hyaluronidase for 2-3 min. For parthenogenetic activation, oocytes were activated by 2 direct pulses of 140 V/mm for 50 s in 0.28 mol/L mannitol supplemented with 0.1 mmol/L $MgSO_4$ and 0.05 mmol/L $CaCl_2$ After 3 h of culture in North Carolina State University (NCSU) 23 medium containing 7.5 g/ml cytochalasin B (CB, Sigma), embryos were washed three times in NCSU 23 medium with 0.4 % (w/v) BSA and cultured in the same medium for 24h at 39℃ in an atmosphere of 5% CO_2 and 95% air.

2.2 Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) assay

Fragmented blastocysts were washed three times in PBS (pH 7.4) containing polyvinylpyrolidone (PVP, 1 mg/ml). This was followed by fixation in 3.7% paraformaldehyde in PBS for 1 h at room temperature (RT). After fixation, the embryos were washed in PBS/PVP and permeabilized by incubation in 0.3% Triton X-100 for 1 h at RT. The embryos were then washed twice in PBS/PVP and incubated with fluorescein-conjugated dUTP and the terminal deoxynucleotidyl transferase enzyme (Roche, USA) in the dark for 1 h at 37℃¡ After counterstaining with 50 g/ml RNase A in 40 g/ml propidium iodide (PI) for 1 h at 37℃¡to label all nuclei, embryos were rinsed in PBS/PVP, mounted with slight coverslip compression and examined under Olympus fluorescence microscope[10].

2.3 Semi-Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Embryos were cultured (from 30 h) in vitro and harvested at the blastocyst stage on day 7 or produced in vivo. Single embryos were washed in Ca^{2+} and Mg^{2+}-free PBS, snap frozen in liquid nitrogen, and stored at−70℃. messenger RNA was extracted using the Dynabeads mRNA Direct Kit (Dynal Asa, Oslo, Norway) according to the manufacturers instructions. In all experiments, beta-actin was used as an internal standard. First, standard cDNA synthesis was achieved by reverse transcription of the RNA by using the random hexamers and the Superscript reverse transcriptase enzyme (Invitrogen Co., Grand Island, NY). The mRNAs of Bcl-xL, Bak and beta-actin were then detected by RT-PCR with specific primer pairs (Table 1) using reagents supplied in a Taq DNA polymerase kit (Takara Korea Biomedical Inc., Seoul, Korea). The PCR products were visualized under ultraviolet light on 1.5% agarose (Invitrogen Co., Grand Island, NY) gels in 1X TAE buffer containing 1 μ g/ml ethidium bromide (Sigma). The intensity of each band was assessed by densitometry using an image analysis program (Lab Works; UVP Inc., Upland, CA). The relative amount of each mRNA species was calculated by dividing the intensity of the bands by the intensity of the corresponding beta-actin band[11].

2.4 Experimental Design and Embryo Culture

To obtain homogenous embryos, optimal 2-cell stage parthenotes were selected 24h after activation. The embryos were randomly allocated into experimental groups.

Experiment 1 examined the effect of different protein supplements on porcine parthenotes development. Diploid parthenotes were recovered after 24 h of culture in NCSU 23 medium containing 0.4% BSA, Presumptive 2-cell stage (30 h) embryos were collected and washed three times in NCSU 23 medium without (control) or with 0.1% PVA (w/v, P-1763, Sigma), 10% FBS (v/v, 16140-071, Gibco), 0.4% BSA (w/v, A-8806, Sigma), 0.1% PVA added 10 μ g/ml murine EGF (Sigma, St. Louis, USA) or 0.4% BSA added 10 ng/ml murine EGF and then randomly cultured in the same medium containing none (control), 0.1% PVA, 10% FBS, 0.4% BSA, 0.1% PVA + 10 ng/ml EGF or 0.4% BSA + 10 μ g/mll EGF. The embryos were cultured for 7 days at 39℃ and 5 % CO_2 in air. On day 7, development to blastocysts were recorded.

Experiment 2 was conducted to determine the effect of protein supplements on the apoptosis and total cell numbers in the blastocysts. In vitro cultured embryos obtained as described in experiment 1 were harvested at the blastocyst stage on day 7 and then used in the TUNEL assay of apoptosis. Total nuclei numbers in these embryos were also counted.

Experiment 3 was conducted to evaluate the effect of protein supplements on Bcl-xL and Bak gene expression in the porcine parthenotes and in vivo produced embryos. Presumptive diploid parthenotes were obtained as described in Experiment 1 were harvested at the blastocyst stage on day 7, washed in PBS and stored at −70℃ before RT-PCR.

2.5 Statistical Analysis

The scores in stage of blastocyst, mean cell number of blastomeres, percentage of apoptosis and relative abundance of gene expression were subjected to analysis of variance using the general linear model (PROC-GLM) in SAS program (Anon, 1992). When the significance of

[Table 1] Primer sequences and cycling conditions used in RT-PCR

Genes	Genbank accession No.	Primer sequence	Position in sequence	Cycle number/ /Annealing temperature	Product size (base pairs)
β-actin	X04751	5´GCAGCCACGGTGGCGAGTAT 3´GTGGGACAGGAGCTTGAAAT	241-260 555-657	35/55℃	257
Bcl-xL	AF216205	5´GGAGCTGGTGGTTGACTTTC 3´CTAGGTGGTCATTCAGGTAAGG	30-49 527-547	35/55℃	518
Bak	AJ001204	5´CTAGAACCTAGCAGCACCAT 3´CGATCTTGGTGAAGTACTC	46-65 178-196	35/55℃	151

the main effects was detected in each experimental parameter, the treatment effects were compared by the least square method. Differences of $p<0.05$ were considered significant.

3. RESULTS

3.1 Effect of Different Supplements on Parthenotes Development (Experiment 1)

More 2-cell embryos develop into morulae and blastocyst at day 7 when BSA was present than its absent ($P<0.01$, Table 2). However, when absence of BSA in the medium, FBS, PVA and PVA+EGF did not significantly increase development of porcine diploid parthenotes.

3.2 Effect of Different Supplements on Cell Numbers and Apoptosis (Experiment 2)

The results of Experiment 2 are presented in Table 3. The mean cell number of blastocysts in the control, PVA, PVA+EGF and BSA groups were similar when cultured from 2-cell parthenotes However, in the presence of FBS, the total cell number decreased significantly decreased at day 7 ($p<0.01$), and EGF treatment enhanced total cell numbers at the blastocysts stage in the NCSU 23 medium supplemented with BSA ($p<0.05$).

Apoptotic cells in individual embryos were measured by the TUNEL assay. There was no difference in apoptosis (fragmented cell number/total cell number) of blastocysts in the control and PVA, PVA+EGF or BSA-derived embryos. However addition of FBS increased the percentage of apoptotic cells ($p<0.05$), but in the presence of BSA, EGF significantly reduced the degree of apoptosis in the blastocysts ($p<0.001$).

3.3 Effect of Different Supplements on Apoptotic Gene Expression (Experiment 3)

To investigate whether different supplements can modulate mRNA expression of apoptotic-related genes in diploid porcine parthenotes developing in vitro, mRNA was prepared from single blastocysts cultured in the NCSU 23 media containing different supplements. Gene expression of Bcl-xL and Bak were examined by RT-PCR analyssis (Table 4). In the PVA-, PVA+EGF and BSA-supplemented medium, the relative abundance of

[Table 2] Developmental ability of porcine parthenotes after 7 days when cultured in NCSU 23 medium containing supplements

Supplement To medium	No. of embryos examined (r)	Percentage of embryos developed to	
		Morulae(%)	Blastocysts(%)
Control(none)	350 (5)	28.0±2.1 [b]	35.9±2.4 [b]
FBS	352 (5)	27.6±2.3 [b]	35.5±3.1 [b]
PVA	356 (5)	28.2±2.0 [b]	39.3±2.4 [b]
PVA+EGF	351 (5)	28.7±2.8 [b]	40.9±3.4 [b]
BSA	359 (5)	48.5±2.1 [a]	60.6±2.4 [a]
BSA+EGF	355 (5)	47.8±2.4 [a]	61.6±3.1 [a]

[Table 3] Number of cells per blastocyst and apoptosis at day 7 when cultured in NCSU 23 medium containing supplements

Supplement To medium	No. of embryos examined (r)	Cell numbers	Percentage of apoptosis
Control(none)	68 (4)	39.8±3.6 [b]	4.7±0.7 [b]
FBS	64 (4)	4.9±2.3 [c]	7.3±0.4 [a]
PVA	65 (4)	40.6±2.9 [b]	4.5±0.5 [b]
PVA+EGF	64 (4)	40.2±2.1 [b]	4.4±0.7 [b]
BSA	68 (4)	46.1±2.6 [b]	4.7±0.6 [b]
BSA+EGF	66 (4)	61.8±2.1 [a]	2.1±0.6 [c]

[Table 4] Relative abundance of mRNA expression in porcine parthenotes at day 7 when cultured in the NCSU 23 medium containing supplements

Supplement To medium	No. of embryos examined (r)	Genes	
		Bcl-xL	Bak
Control(none)	8 (8)	0.24±0.02 [b]	0.14±0.01 [b]
FBS	8 (8)	0.18±0.02 [c]	0.34±0.02 [a]
PVA	8 (8)	0.24±0.03 [b]	0.17±0.02 [b]
PVA+EGF	8 (8)	0.25±0.02 [b]	0.16±0.03 [b]
BSA	8 (8)	0.26±0.03 [b]	0.16±0.02 [b]
BSA+EGF	8 (8)	0.44±0.04 [a]	0.17±0.02 [b]

Bcl-xL mRNA expression was similar to control-derived blastocysts and higher than FBS-derived embryos ($P<0.05$). However in the presence of BSA, EGF enhanced relative abundance of Bcl-xL mRNA expression ($p<0.01$).

There was no difference in Bak mRNA expression in control, PVA, PVA+EGF, BSA and BSA+EGF derived embryos, but significantly enhanced in FBS-derived embryos ($p<0.005$).

4. DISCUSSION

In the present study we demonstrated the effects of exogenous protein supplements on porcine blastocyst production. The experiments were performed with embryos of homogeneous quality by selecting good quality porcine diploid parthenotes at the 2-cell stage. We found that porcine presumptive diploid parthenotes at 30h after activation developed to the blastocyst stage at a relatively high rate in the presence of BSA as compared to those in the presence of either PVA or none-supplement control. However, BSA did not increase the cell numbers of blastocysts. We found that BSA increased the cell numbers of the cultured blastocysts but that this effect was manifested only when EGF was present. EGF on its own had no effect. That the effect of EGF is observed only when BSA is present suggests a synergic effect between EGF and BSA on the cell numbers of the cultured blastocysts. EGF has been shown to stimulate both the cellular proliferation and differentiation of various somatic cells [12] and in the mouse, EGF receptor mRNA and protein levels are increased after the 4-cell stage[3]. Similarly, porcine embryos express the EGF receptor mRNA during the morula and blastocyst stages[3]. Thus, exogenous EGF may act as a mitogen during early embryo development by binding to its cognate receptor. Our study showed that EGF decreased apoptosis, but only in combination with BSA. EGF or BSA by itself had no effect on apoptosis. These observations are in line with the effect of BSA and EGF on cell numbers, as blastocyst cell numbers are the end result of a combination of cell proliferation and cell death processes. In the present study FBS decreased cell numbers in blastocysts cultured from 24h. The ratio of TUNEL-labeled DNA to total DNA area of FBS-derived blastocysts was greater than in its absence medium-derived counterparts (7% vs. 4%). This result is consistent with previous results in cattle. Exposure of early cattle embryos (2 to 8 cell) to FBS significantly reduced the formation of blastocoel, blastocysts cell numbers[13]. Supporting the idea that EGF reduces apoptosis and FBS enhances apoptosis are our observations on the expression of two apoptosis-related genes during blastocyst development in vitro. Supporting the idea that EGF reduces apoptosis induced by BSA factors are our observations on the expression of two apoptosis-related genes during blastocyst development in vitro. In the present study, we found that EGF together with BSA enhanced Bcl gene expression, while EGF and BSA on their own had no such effect. We also found FBS decreased Bcl-xL gene expression in blastocysts developing in vitro. This observation may reflect the ability of FBS to reduce the apoptotic process in pig blastocysts. Bcl-xL is a very potent cell death suppressor. In developing embryos increased expression of cell death inducers genes would be inhibited cell protector expression (e.g., Bcl-xL) would be increased, augmenting maternal stores (e.g., Bcl-xL,[14]. Pro-apoptotic gene Bak was higher in blastocysts produced in the presence of

FBS than produced in its absence embryos, which would be consistent with the notion that mRNA levels for this gene are higher in bad-quality embryos.

In conclusion, our data indicate that conditions of supplementing PVA, FBS, BSA or EGF in culture medium, affect on developing, cell numbers and apoptosis. BSA enhanced in vitro development of porcine diploid parthenotes, while FBS reduce embryo viability. However, the addition of EGF to the culture medium in the presence of BSA reduced apoptosis and increased the cell number of porcine presumptive diploid parthenotes. The effect of EGF and FBS are likely mediated by their ability to regulate Bcl-xL and Bak genes expression, which affect cell numbers.

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Animal Reproductive Physiology